1   # Conserved type VI secretion regulation in diverse *Vibrio* species by

2   # the regulatory proteins TfoX and TfoY

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17

18   **Originality-Significance Statement**

19   This work provides new insight into the regulatory circuits involved in type VI secretion in

20   diverse *Vibrio* species. Specifically, it is the first study to compare the effects of the two

21   regulatory proteins TfoX and TfoY on the primary or secondary type VI secretion systems of

22   non-cholera vibrios. Importantly, this work also shows that decreased c-di-GMP levels in *V.*

23   *parahaemolyticus* lead to TfoY production without changing *tfoY* transcript levels, thereby

24   indirectly linking TfoY production to surface sensing.

25   **Summary**

26   Bacteria of the genus *Vibrio* are common members of aquatic environments where they compete

27   with other prokaryotes and defend themselves against grazing predators. A macromolecular

28   protein complex called the type VI secretion system (T6SS) is used for both purposes. Previous

29   research showed that the sole T6SS of the human pathogen *V. cholerae* is induced by

30   extracellular (chitin) or intracellular (low c-di-GMP levels) cues and that these cues lead to

31   distinctive signalling pathways for which the proteins TfoX and TfoY serve as master regulators.

32   In this study, we tested whether the TfoX- and TfoY-mediated regulation of T6SS was conserved

33   in non-cholera species, and if so, how these regulators affected the production of individual

34   T6SSs in double-armed vibrios. We show that, alongside representative competence genes, TfoX

35   regulates at least one T6SS in all tested *Vibrio* species. TfoY, on the other hand, fostered motility

36   in all vibrios but had a more versatile T6SS response in that it did not foster T6SS-mediated

37   killing in *V. fischeri* while it induced both systems in *V. alginolyticus*. Collectively, our data

38   provide evidence that the TfoX- and TfoY-mediated signalling pathways are mostly conserved in

39   diverse *Vibrio* species and important for signal-specific T6SS induction.

Introduction

40

41   Secretion systems that enable the export of macromolecules across membranes are often

42   associated with pathogenesis in Gram-negative bacteria. However, these machineries and their

43   substrates also play important roles in the interactions between bacteria and other

44   microorganisms in their natural environment (Costa *et al.*, 2015; Green and Mecsas, 2016). An

45   important secretion system, the type VI secretion system (T6SS), was first described in *Vibrio*

46   *cholerae* and is a syringe-like killing device involved in virulence and inter-bacterial warfare

47   (Pukatzki *et al.*, 2006; Ho *et al.*, 2014; Russell *et al.*, 2014). T6SSs are present in ~25% of

48   sequenced Gram-negative bacteria, including pathogenic and non-pathogenic species, and are

49   defined by a set of 13 core components (Das and Chaudhuri, 2003; Bingle *et al.*, 2008; Boyer *et*

50   *al.*, 2009). These components build a macromolecular complex that shares both structural and

51   functional homology with contractile bacteriophage tails, such as in the phage T4 (Leiman *et al.*,

52   2009; Basler, 2015; Taylor *et al.*, 2018). Briefly, a tail-like structure, which is composed of

53   hemolysin coregulated protein (Hcp) hexamers and sharpened by spike-like tip proteins (VgrG

54   and PAAR), is surrounded by a contractile sheath structure and polymerizes onto a membrane-

55   spanning multi-protein complex. When the sheath contracts, the effector-decorated tip complex

56   is propelled outwards together with the inner tube to puncture the membrane of a neighboring

57   target cell and inject toxic effector proteins (reviewed by (Zoued *et al.*, 2014; Cianfanelli *et al.*,

58   2016). Each T6SS encodes its own sets of variable effector proteins that determine the specificity

59   and the biological function of the system, as they can impact both eukaryotic host cells and

60   bacterial competitors. To prevent self-intoxication and to kin-discriminate siblings, bacteria

61   produce effector-specific neutralizing immunity proteins, which are frequently encoded adjacent

62   to the effector gene in a bicistronic unit (Durand *et al.*, 2014; Alcoforado Diniz *et al.*, 2015).

63   Studying the various T6SS systems and their regulation is therefore important, as such

64 knowledge sheds light onto the evolution of vibrios in response to adapting niches and their

65 interaction with environmental hosts.

66 A comparison of the various T6SS-associated across *Vibrio* species indicate that there must be

67 multiple different functions and regulatory mechanisms associated with the system(s). The

68 structural components of the T6SS are mostly encoded within larger gene clusters, which are

69 variable with respect to genetic content and organization. The genes coding for the secreted core

70 components (Hcp, VgrG, PAAR) are often found in smaller auxiliary clusters together with the

71 genes that encode the effectors-immunity pairs (Boyer *et al.*, 2009). Many bacterial genomes

72 encode more than one T6SS, which likely serve different needs (Boyer *et al.*, 2009). Accordingly,

73 bacteria employ a wide variety of regulatory mechanisms, from common cues to specific

74 regulatory cascades, to ensure appropriate expression of T6SS genes (Miyata *et al.*, 2013), which

75 likely vary within species when multiple T6SS systems are present.

76 *V. cholerae* O1 El Tor strains, which are responsible for the current 7th pandemic of cholera

77 (further referred to as pandemic strains), harbor a single T6SS. In contrast, many non-cholera

78 *Vibrio* species encode several T6SSs, such as clinical isolates of *V. parahaemolyticus* and

79 environmental isolates of *V. vulnificus,* both of which carry two T6SSs (Yu *et al.*, 2012; Church

80 *et al.*, 2016). The genome of the squid symbiont *V. fischeri,* on the other hand, codes for one

81 T6SS even though a recent study by Speare *et al.* identified a secondary T6SS in several *V.*

82 *fischeri* isolates that fostered niche domination within the squid's light organ (Speare *et al.*,

83 2018).

84 The T6SS of pandemic *V. cholerae* is silent under standard laboratory conditions (Pukatzki *et*

85 *al.*, 2006). In contrast to pandemic strains, the O37 serogroup strains V52 and ATCC25872 are

86 toxigenic but non-pandemic and their T6SS is constitutively activated, as is the case for most

87 environmental isolates (Pukatzki *et al.*, 2006; Unterweger *et al.*, 2012; Bernardy *et al.*, 2016;

88 Van der Henst *et al.*, 2018). To explain the differences in T6SS activity and to provide insight

89   into the T6SS's biological functions, it is therefore important to understand the underlying

90   regulatory pathways in pandemic *V. cholerae* strains. Accordingly, several minor and major

91   T6SS regulators have been identified in *V. cholerae* (Joshi *et al.*, 2017) by changing their

92   abundance through deletion or forced expression of the respective genes, which resulted in

93   changes in T6SS gene expression or T6SS activity. Two of these major regulators, TfoX and

94   TfoY (Borgeaud *et al.*, 2015; Metzger *et al.*, 2016), contain TfoX-like N- and C-terminal

95   domains, and proteins containing such domains are usually annotated as regulators of natural

96   competence for transformation due to their presence in the competence regulator Sxy of

97   *Haemophilus influenzae* (Redfield, 1991) and TfoX in *V. cholerae* (Meibom *et al.*, 2005). We

98   recently showed that TfoX induces the T6SS in parallel with the competence machinery at a high

99   bacterial cell density (measured by quorum sensing [QS] and signaled through its master

100   regulator, HapR) on chitinous surfaces, leading to T6SS-mediated killing followed by the

101   absorption of prey-released DNA (Borgeaud *et al.*, 2015). Coupling kin-discriminating neighbor

102   killing and competence fosters horizontal gene transfer and, ultimately, evolution (Veening and

103   Blokesch, 2017).

104   Interestingly, *V. cholerae* and most other members of the genus *Vibrio* (vibrios) contain a

105   TfoX homolog named TfoY (Pollack-Berti *et al.*, 2010; Metzger *et al.*, 2016). However, despite

106   the homology to TfoX and its common annotation as a competence regulator, TfoY is neither

107   essential nor sufficient for competence induction in *V. cholerae* (Metzger *et al.*, 2016). TfoY also

108   induces the T6SS of *V. cholerae*, though the TfoX and TfoY regulons are non-overlapping, with

109   TfoX co-inducing competence and TfoY orchestrating a defensive reaction. This defensive

110   reaction includes enhanced motility and the co-production of the T6SS with additional

111   extracellular enzymes, some of which are known to intoxicate amoebal predators (Metzger *et al.*,

112   2016; Van der Henst *et al.*, 2018). Moreover, TfoX is naturally produced upon growth on

113   chitinous surfaces (Meibom *et al.*, 2005), while TfoY production is inhibited by c-di-GMP, an

114   secondary messenger in bacteria, such that it is only present at low intracellular c-di-GMP levels

115   (Inuzuka *et al.*, 2016; Metzger *et al.*, 2016).

116   Given the presence of TfoX and TfoY in many vibrios, we aimed to understand their role in

117   non-cholera species. We show that the T6SS activation is highly conserved across vibrios, with

118   the majority of tested species concomitantly inducing natural competence and motility when

119   TfoX and TfoY, respectively, are produced. Interestingly, we observed different scenarios when

120   comparing those *Vibrio* species that encode more than one T6SS, with TfoX and TfoY not

121   fulfilling the same inducing role for all T6SSs. We therefore conclude that some vibrios

122   specifically induce one or the other T6SS under different environmental conditions driven by

123   either TfoX or TfoY, while other species combine forces to simultaneously induce both T6SSs.

124

125   **Results and discussion**

126   ***Conservation of T6SS production by TfoX and TfoY in pandemic isolates of V. cholerae***

127   Since its discovery in a non-pandemic strain of *V. cholerae*, it has been reported that the T6SS of

128   pandemic strains is silent under standard laboratory conditions (Pukatzki *et al.*, 2006). We

129   recently showed that the two regulatory proteins TfoX and TfoY foster T6SS production and,

130   accordingly, inter-bacterial competition in pandemic *V. cholerae* (Borgeaud *et al.*, 2015; Metzger

131   *et al.*, 2016). The previous study that addressed TfoY, however, was based on a single strain of *V.*

132   *cholerae*, namely strain A1552. This human isolate originated from food-borne transmission on

133   an airplane returning to the US from Peru/South America (Blokesch, 2012a) and therefore

134   belongs to the West-African South American (WASA) lineage (precisely, the LAT-1 sublineage)

135   of the currently ongoing seventh cholera pandemic (Mutreja *et al.*, 2011; Domman *et al.*, 2017).

136   As strain-specific differences are frequently reported, this study was designed to first confirm the

137   generality of TfoX- and TfoY-dependent T6SS activation in pandemic strains of *V. cholerae*. To

138   do this, we tested five isolates from three different countries (Bangladesh, Peru, and Bahrain) for

139   T6SS-dependent killing of *Escherichia coli*. Notably, we used a derivative of the first sequenced

140   isolates of *V. cholerae*, pandemic strain N16961 (Heidelberg *et al.*, 2000), in which the

141   frameshift mutation in the quorum-sensing regulator gene *hapR* was repaired (Kühn *et al.*, 2014).

142   As demonstrated in Figure S1, all strains behaved similarly to A1552 upon TfoX or TfoY

143   production. We therefore suggest that the dual control over T6SS by these two regulators is

144   conserved among pandemic *V. cholerae*.

145

146   ***Functionality of TfoX and TfoY homologs from other Vibrio species in V. cholerae***

147   Pollack-Berti and colleagues showed that TfoX and TfoY existed in all fully sequenced

148   *Vibrionaceae* (Pollack-Berti *et al.*, 2010), so we wondered whether these homologs would act in

149   a comparable manner in non-cholera vibrios despite the fact that these *Vibrio* species are often

150   adapted to different environmental niches (Le Roux and Blokesch, 2018) and their homologous

151   proteins might therefore serve different, adaptation-specific functions. We therefore compared

152   the protein sequences of TfoX and TfoY from four different *Vibrio* species, *V. cholerae, V.*

153   *parahaemolyticus, V. alginolyticus,* and *V. fischeri,* with the additional species chosen because

154   previous studies had already described the genetic organization of their T6SS (Salomon *et al.*,

155   2013; Salomon *et al.*, 2014; Salomon *et al.*, 2015; Speare *et al.*, 2018). Moreover, these

156   additional species have isolates that contain two T6SSs. We therefore considered the possibility

157   that TfoX and TfoY, which are produced under vastly different conditions in *V. cholerae*

158   (Metzger and Blokesch, 2016), might be specialized for the induction of a single T6SS in those

159   organisms that harbor several systems. By aligning the protein sequences, we observed a high

160   level of protein conservation for TfoX and TfoY, with a sequence identity of 68%/66%,

161   67%/66%, and 59%/67% when the homologous proteins of *V. parahaemolyticus*

162   (TfoX$_{Vp}$/TfoY$_{Vp}$), *V. alginolyticus* (TfoX$_{Va}$/TfoY$_{Va}$), and *V. fischeri* (TfoX$_{Vf}$/TfoY$_{Vf}$) were

163   compared to the respective homologs of *V. cholerae* (Fig. 1A). Given these identity values, we

164   concluded that the TfoX of *V. parahaemolyticus* and *V. alginolyticus* were more closely related

165   to the homologous protein of *V. cholerae*, whereas TfoX from *V. fischeri* diverged the most,

166   which is in accordance with the phylogenetic distances between the species (Fig. 1A).

167   Given the high level of protein conservation, we next tested whether the protein homologs

168   from the non-cholera vibrios could function in *V. cholerae*. For this purpose, we cloned the

169   respective genes preceded by an arabinose-inducible promoter (P_{BAD}) onto a site-specific

170   transposon (mini-Tn7) together with the gene that encodes the arabinose-responsive regulatory

171   protein AraC (Table S1). Next, we grew the genetically engineered strains and the parental WT

172   strain in the presence of arabinose (as inducer), isolated their RNA, and then quantified the

173   transcript levels of representative T6SS-encoding genes. As shown in Fig. 1B, heterologous

174   production of the diverse TfoX proteins resulted in increased T6SS transcript levels compared to

175   the uninduced conditions, though the level of induction was slightly lower than the production of

176   *V. cholerae*'s own TfoX, especially for the most diverging protein variant from *V. fischeri*

177   ($TfoX_{Vf}$). We confirmed these data at the protein level through the detection of the inner tube

178   protein of the T6SS, Hcp (Fig. 1C). Consistent with the production of Hcp and the increase of the

179   T6SS transcript level, these strains were able to kill *E. coli* in an interbacterial competition assay,

180   with the least predatory behavior observed for the most divergent $TfoX_{Vf}$-carrying variant (Fig.

181   1D).

182   TfoX of *V. cholerae* was first identified as the master regulator of natural competence for

183   transformation (Meibom *et al.*, 2005; Metzger and Blokesch, 2016). The TfoX-induced

184   competence regulon includes those genes that encode the structural components of the DNA-

185   uptake machinery, which primarily consists of a central pilus structure (encoded by *pil* genes;

186   Seitz and Blokesch, 2013) and the DNA binding protein ComEA that is essential for reeling

187   DNA across the outer membrane and into the periplasmic space (Seitz and Blokesch, 2014; Seitz

188   *et al.*, 2014; Matthey and Blokesch, 2016). We therefore tested whether the TfoX variants from

- 8 -

189   the other *Vibrio* species would likewise foster competence-gene expression, which was indeed

190   the case (Fig. 1E). Compared to the other variants, the more diverse $TfoX_{Vf}$ did not induce the

191   same high transcript levels of *comEA*, which, accordingly, also led to lower transformation

192   frequencies (Fig. 1F). Notably, *comEA* expression in *V. cholerae* is regulated by an intermediate

193   transcription factor, QstR, which is dependent on the production of both TfoX and HapR (Lo

194   Scrudato and Blokesch, 2013; Jaskólska *et al.*, 2018). HapR is the master regulator of the QS

195   system, meaning that *comEA* expression is dependent on a functional QS system and on high cell

196   density. As *V. fischeri* - but not the other tested *Vibrio* species - lacks a QstR homolog, we

197   suggest that $TfoX_{Vf}$ induces *comEA* at lower levels, as it is adapted to the more diverse QS circuit

198   of *V. fischeri* and the absence of QstR (Milton, 2006; Verma and Miyashiro, 2013).

199   We next tested the functionality of the TfoY homologs in *V. cholerae* by first testing the

200   ability of the TfoY variants to foster natural transformation, given that these proteins are

201   frequently annotated as "DNA transformation protein TfoX" or "TfoX-family DNA

202   transformation protein" due to their similarity to TfoX. However, no transformants were detected

203   upon the production of any of these proteins, indicating their inability to induce natural

204   competence in *V. cholerae* (Fig. 1F). Conversely and consistent with the high sequence similarity

205   of the proteins from the four organisms, all TfoY variants functioned at the same level as the *V.*

206   *cholerae* TfoY to increase T6SS transcript levels (Fig. 1G), to induce Hcp protein production

207   (Fig. 1H), and, consequently, to foster interbacterial killing of *E. coli* (Fig. 1I). In addition, the

208   four TfoY variants were able to induce motility as a T6SS-independent phenotype, as visualized

209   on swarming agar plates and quantified in Fig. 1J. Taken together, these data indicate that TfoX

210   and TfoY are conserved proteins among these four *Vibrio* species and that the TfoY variants can

211   fully replace the *V. cholerae* innate protein, while the TfoX variants are functionally replaceable

212   at high levels for $TfoX_{Vp}$/$TfoX_{Va}$ and intermediate levels for $TfoX_{Vf}$.

213

214   ***TfoX but not TfoY induces V. fischeri's primary T6SS***

215   While the complementation assay in *V. cholerae* described above provided a first insight into the

216   conservation of TfoX and TfoY from diverse vibrios by indicating their similarity to those of *V.*

217   *cholerae*, the experiments did not tell us whether these proteins would be involved in similar

218   phenotypes as in *V. cholerae* in their native hosts. We therefore genetically engineered the

219   different *Vibrio* species to place inducible copies of their own *tfoX* and *tfoY* genes onto a mini-

220   Tn7 transposon that was then integrated into their own genomes (Table S1). Next, we assessed

221   the different phenotypes under uninduced and induced conditions. For *V. fischeri* strain ES114

222   (Table S1), we showed that TfoX production led to a high induction of T6SS transcripts, while

223   TfoY only partially induced a subset of T6SS genes (Fig. 2A). Consistent with these expression

224   data, we witnessed the T6SS-mediated killing of *E. coli* only upon TfoX induction, while TfoY

225   induction did not change the number of surviving co-cultured prey bacteria (Fig. 2B).

226   Pollak-Berti *et al*. showed that TfoX was required for natural competence for transformation

227   in *V. fischeri* (Pollack-Berti *et al.*, 2010)*,* as is the case for *V. cholerae* (Meibom *et al.*, 2005).

228   We therefore measured relative transcript levels of representative competence genes in this

229   organism and confirmed their induction upon TfoX production (Fig. 2C). Surprisingly, while we

230   only observed a mild induction of *comEA,* we witnessed an almost 10-fold upregulation of *litR,*

231   which encodes the main QS regulator LitR (homolog of *V. cholerae*'s HapR). This phenotype

232   differs from what is known about competence regulation in *V. cholerae* in which case *hapR*

233   transcript levels are left unchanged upon TfoX induction (Meibom *et al.*, 2005; Metzger and

234   Blokesch, 2016). We therefore speculate that an increase in LitR levels can compensate for the

235   lack of the intermediate regulator QstR in *V. fischeri*. Indeed, the QstR of *V. cholerae* is required

236   for competence-mediated DNA uptake in two ways: i) through direct and in-direct induction of

237   certain competence genes, such as *comEA*, as described above; and ii) due to its ability to

238   downregulate the *dns* gene (Lo Scrudato and Blokesch, 2013; Jaskólska *et al.*, 2018) encoding

239   for the extracellular nuclease Dns, which degrades transforming material outside the cell and

240   within the periplasmic space (Blokesch and Schoolnik, 2008; Seitz and Blokesch, 2014). Notably,

241   the HapR protein of *V. cholerae* also silences *dns* expression, but only partially in the absence of

242   QstR (Lo Scrudato and Blokesch, 2013). It is therefore likely that the increased production of

243   LitR upon TfoX production in *V. fischeri* compensates for the absence of QstR to ensure

244   sufficient repression of *dns,* which, ultimately, would allow DNA uptake to occur. It is not clear,

245   however, why the *comEA* transcripts were not induced to higher levels, though we recently

246   demonstrated for *V. cholerae* that the *comEA* gene requires an additional but so far unidentified

247   regulatory input apart from TfoX and QstR (Jaskólska *et al.*, 2018). Indeed, we demonstrated

248   that artificial QstR production was sufficient to induce the T6SS genes in this organism at

249   comparable levels as the upstream regulatory protein TfoX, even in the absence of HapR, while

250   the *comEA* transcript levels were only partially increased compared to a non-induced WT strain.

251   We therefore hypothesize that this input signal might be conserved in *V. fischeri*, though missing

252   under the tested conditions. This would lead to a high overall competence-gene induction upon

253   TfoX production but lower *comEA* transcripts as compared to *V. cholerae* (Fig. 1E).

254   Lastly, we determined that the motility of *V. fischeri* changed upon TfoY production (Fig. 2D),

255   even though the difference between the TfoY-induced and TfoY-uninduced state was not as

256   pronounced as for *V. cholerae* (Fig. 1J). We argue that this might be caused by a basal

257   production level of TfoY in this organism that does not rely on the artificial induction. A higher

258   basal TfoY level would result in a higher basal motility, which would therefore appear as a less

259   pronounced induction upon additional TfoY production. There was also a decrease in motility

260   observable upon TfoX production (Fig. 1D), which is consistent with the idea of a basal TfoY

261   level, as the expression data showed that TfoX significantly lowered the *tfoY* transcript levels

262   (Fig. 2A). Likewise, TfoY production lowered *tfoX* transcript levels (Fig. 2A), suggesting that

263   both proteins work independently of each other and are mutually exclusive in *V. fischeri*. This

264 finding contradicts a previous study in which the authors concluded that TfoY was required for

265 efficient TfoX-mediated transformation (Pollack-Berti *et al.*, 2010). Notably, both studies used

266 different conditions (e.g., inducible TfoX/TfoY expression in this study versus a transposon-

267 inserted mutant of *tfoY* (Pollack-Berti *et al.*, 2010)) and further studies are therefore required to

268 conclusively show if TfoY is indeed involved in natural transformation in *V. fischeri* or not, as

269 we suggest based on the herein-described data.

270

271 ***TfoX and TfoY have a different impact on T6SS induction in V. alginolyticus***

272 While *V. cholerae* strains and the examined *V. fischeri* strain ES114 contain only a single T6SS,

273 several other *Vibrio* species contain more than one. We therefore asked whether, and if so how,

274 TfoX and TfoY affect the production of different T6SSs within the same organism. To address

275 this question, we first tested the contribution of these regulators to the two T6SSs (T6SS1 and

276 T6SS2) of *V. alginolyticus* in an *E. coli*-killing assay using wildtype (WT) *V. alginolyticus* or its

277 *hcp1* (referred to as ΔT6SS1 throughout the text), *hcp2* (referred to as ΔT6SS2 throughout the

278 text), or double mutant as a predator. Consistent with a previous report by Salomon *et al*.

279 (Salomon *et al.*, 2015), WT *V. alginolyticus* did not show interbacterial predation at 37°C on

280 standard LB medium (e.g., without additional salt; Fig. 3A). Upon production of TfoX or TfoY,

281 however, predation by the WT was significantly increased. This effect was less pronounced but

282 reproducible in the strain that only carried the complete T6SS2 gene set (ΔT6SS1), while a strain

283 that only carried a complete T6SS1 gene set (ΔT6SS2) was not able to kill the *E. coli* cells,

284 indicating that T6SS1 is non-functional at this temperature. In contrast, both systems were

285 inducible at 30°C, with T6SS1 specifically induced by TfoY and not TfoX, while T6SS2 was

286 responsive to both regulatory proteins (Fig. 3B). Basic killing activity was also observed in the

287 WT and the mutant lacking T6SS1 but not in a mutant lacking T6SS2, suggesting that T6SS2 is

288 slightly induced under the tested conditions but can be further boosted upon TfoX and TfoY

289   production (Fig. 3B). While this finding seems at first glance to contradict a previous study that

290   reported basal activity for T6SS1 (Salomon *et al.*, 2015), it should be noted that the experimental

291   setup differed in that we used an assay in which the *Vibrio* are tested at high cell density, as was

292   previously developed for *V. cholerae* (Borgeaud *et al.*, 2015), while Salomon *et al.* first diluted

293   the cells to low densities. The rationale behind our experimental setup was the knowledge that

294   the TfoX-mediated pathway requires co-induction by HapR in *V. cholerae* (homologs are LuxR

295   in *V. alginolyticus* and OpaR in *V. parahaemolyticus*) (Lo Scrudato and Blokesch, 2012; Metzger

296   and Blokesch, 2016; Jaskólska *et al.*, 2018), which we assumed to be a conserved feature in other

297   *Vibrio* species (see data below for *V. parahaemolyticus*).

298   In addition to T6SS activation, we also tested the level of competence genes transcripts and

299   bacterial motility upon TfoX and TfoY induction. As shown in Fig. 3C, a significant TfoX-

300   mediated induction of representative competence genes occurred, indicating the protein's

301   conserved action. Notably, the *comEA* transcript induction levels again turned out to be

302   unexpectedly low compared to the induction levels in *V. cholerae* (Fig. 1E). ComEA plays a

303   primary role in ratcheting the DNA into the periplasmic space in competent bacteria (Seitz *et al.*,

304   2014), and we therefore expect that these low transcript levels would lead to insufficient amounts

305   of the ComEA proteins and defective DNA uptake. We speculate that the additional but so far

306   unknown regulatory input required for *comEA* expression mentioned above (Jaskólska *et al.*,

307   2018) might also be a prerequisite in *V. alginolyticus* and that this pathway might be non-

308   functional in either the strain used in this study or under the tested conditions or both. The

309   motility-inducing phenotype of TfoY, on the hand, was very pronounced in *V. alginolyticus,*

310   strengthening the idea that TfoY protein levels and a high motility are linked throughout the

311   genus *Vibrio,* even in organisms that contain lateral flagella in addition to the polar flagellum

312   (McCarter, 2001).

313

TfoX and TfoY are both dedicated to one specific T6SS in *V. parahaemolyticus*

314

315   Lastly, we tested the contribution of TfoX and TfoY to T6SS regulation in *V.*

316   *parahaemolyticus*. As for *V. alginolyticus*, this bacterium also contains two T6SSs, T6SS1 and

317   T6SS2, which were previously shown to be differentially regulated from each other with respect

318   to temperature, salinity, QS inputs and surface sensing (Salomon *et al.*, 2013). Indeed, T6SS1 of

319   *V. parahaemolyticus* was found to be most active under marine-like conditions and at low cell

320   densities while system 2 was more adapted to lower salt conditions (e.g., LB medium) and higher

321   cell densities (Salomon *et al.*, 2013). Notably, these data on T6SS2 regulation and a previous

322   study that suggested T6SS2 was involved in adhesion to host cells were solely based on the Hcp2

323   protein levels (inside and outside the cells), while interbacterial killing activity was not directly

324   observed for T6SS2 (Yu *et al.*, 2012; Salomon *et al.*, 2013). Indeed, the QS-dependent T6SS2

325   regulation of Hcp production confirmed previous results in *V. cholerae* (Ishikawa *et al.*, 2009).

326   However, despite the fact that pandemic *V. cholerae* produce detectable levels of Hcp at high

327   cell densities *in vitro*, the bacteria are unable to kill prey under such conditions (Pukatzki *et al.*,

328   2006), which can be overcome by TfoX or TfoY production (Metzger *et al.*, 2016). Indeed, we

329   previously demonstrated that upon induction of these two regulators, the Hcp levels increased

330   further, and T6SS-mediated prey killing was observed. Notably, TfoY-mediated T6SS activity in

331   *V. cholerae* occurred independently of HapR, indicating that TfoY acts independently from the

332   QS pathway, in contrast with TfoX-mediated T6SS activation (Borgeaud *et al.*, 2015; Metzger *et*

333   *al.*, 2016).

334   In *V. parahaemolyticus*, both TfoX and TfoY production led to significant *E. coli* killing (Fig.

335   4A). Interestingly, when testing *V. parahaemolyticus* strains that contained only a complete

336   T6SS2 gene set (ΔT6SS1) or only all T6SS1 genes (ΔT6SS2), it was clear that TfoX specifically

337   induced T6SS2, while TfoY solely led to T6SS1-mediated prey killing (Fig. 4A). These data also

338   suggested that the T6SS2 was slightly active in both the WT and the strain lacking T6SS1, even

339 without TfoX/TfoY production. We then determined that low levels of TfoX or TfoY did not

340 cause this basal activity, as deletion of the indigenous genes did not abolish it (Fig. 4B).

341 Interestingly, a recent study showed T6SS2-dependent killing of a *V. parahaemolyticus* strain

342 that lacked a newly identified effector immunity pair (RhsP and RhsPi) by its parental WT strain,

343 even in the absence of TfoX or TfoY induction (Jiang *et al.*, 2018). These data therefore suggest

344 that the basal activity that we observed against *E. coli* as a prey in this study might be much more

345 enhanced in killing non-immune siblings compared to other species or, alternatively, that the

346 experimental conditions were different to the current work and that under such conditions the

347 T6SS2 is highly active. Lastly, we cannot exclude the possibility that strain differences or the

348 domestication of certain strains has caused the difference in basal T6SS activity. Notably, the

349 focus of the current study was on the contribution of TfoX or TfoY to T6SS activity, which we

350 unambiguously demonstrate to enhance T6SS activity in *V. parahaemolyticus*.

351 Examining other TfoX- and TfoY-mediated phenotypes confirmed the increase of

352 competence gene transcripts and enhanced motility, respectively (Fig. 4C and D). However, the

353 *comEA* levels were again unexpectedly low, which explains why we were unable to naturally

354 transform *V. parahaemolyticus* even after TfoX induction (data not shown). The induction levels

355 of the gene encoding *qstR* were comparable with what we observed for *V. cholerae* (Fig. 1E)

356 where TfoX-dependent T6SS induction seemed functional (Fig. 4A), so we hypothesize that the

357 transformation defect was *comEA*-specific, as discussed above, and not based on a general

358 mutation within the QS cascade. Previous studies in different *Vibrio* species described gain-of-

359 function mutations in LuxO, a repressor of HapR/LitR/OpaR synthesis (Gode-Potratz and

360 McCarter, 2011; Kimbrough and Stabb, 2015; Stutzmann and Blokesch, 2016) and clinical *V.*

361 *parahaemolyticus* isolates with such *luxO* mutations were previously described to be locked in

362 a pathogenic state (Kernell Burke *et al.*, 2015). To exclude that this has happened in the strain we

363 were working with, we first sequenced *luxO* and *opaR* and confirmed that both genes were

- 15 -

364   mutation free. Additionally, we deleted *opaR* from the WT strain and compared this strain to its

365   TfoX- or TfoY-inducible derivatives (Table S1) in an *E. coli*-killing assay. As shown in Fig. 4E,

366   the TfoY-induced prey killing was maintained upon deletion of *opaR*, while the TfoX-mediated

367   T6SS activity was abrogated. Since OpaR is produced at high cell density, its deletion blocks

368   QS-dependent signalling. We therefore conclude that the WT strain has a functional OpaR and

369   that TfoX-mediated T6SS induction in *V. parahaemolyticus* requires co-regulation by OpaR,

370   similar to *V. cholerae* (Borgeaud *et al.*, 2015; Metzger *et al.*, 2016).

371   Our data suggest that TfoX induces the T6SS2 in *V. parahaemolyticus*. As previous studies

372   showed that the chitin sensors ChiS, TfoS, and the downstream-regulated small RNA TfoR,

373   which is required for *tfoX* mRNA translation, are highly conserved in diverse *Vibrio* species (Li

374   and Roseman, 2004; Yamamoto *et al.*, 2011; Yamamoto *et al.*, 2014), we suggest that TfoX is

375   exquisitely produced upon growth on chitinous surfaces, as first demonstrated for *V. cholerae*

376   (Meibom *et al.*, 2004; Meibom *et al.*, 2005). The production of TfoY is less understood. In a

377   previous study on *V. cholerae*, we showed that lowered c-di-GMP levels led to the production of

378   TfoY under standard laboratory conditions (e.g., in LB medium) and we and others suggested

379   that this c-di-GMP-dependent control occurred at the translational level (Inuzuka *et al.*, 2016;

380   Metzger *et al.*, 2016). Interestingly, Gode-Potratz *et al.* demonstrated that growth on surfaces

381   lowered the levels of the secondary messenger c-di-GMP in *V. parahaemolyticus* (Gode-Potratz

382   *et al.*, 2011). Consistently, Salomon *et al.* established that surface sensing correlated with Hcp1

383   production in this organism (Salomon *et al.*, 2013). Based on these data, we speculated that

384   surface sensing, TfoY production, and T6SS1 induction might be linked. To address this

385   hypothesis and especially the link between low c-d-GMP levels and TfoY production, we

386   genetically engineered *V. parahaemolyticus* to carry a translational fusion between its indigenous

387   *tfoY* gene and the gene that encodes super-folder GFP (sfGFP). In addition, we included a

388   transcriptional mCherry-encoding reporter gene behind this construct, which, ultimately, allowed

- 16 -

389   us to monitor the transcriptional and translational control of *tfoY*. Next, we incorporated either an

390   empty mini-Tn7 transposon (Tn) or transposons that carried arabinose-inducible genes coding

391   for a c-di-GMP-producing diguanylate cyclase (Tn-*vdcA*) or for a phosphodiesterase (Tn-*cdpA*)

392   into this strain (Table S1). These strains as well as the transposon-deficient parental strain and

393   the WT without any fluorescent protein-encoding genes were then grown in the presence of the

394   inducer arabinose followed by a western blot analysis to visualize the TfoY-sfGFP fusion protein

395   or the transcriptional reporter protein mCherry. Using this approach, we observed that TfoY was

396   produced solely at low ci-di-GMP levels (Fig. 4F). Importantly, based on the comparable levels

397   of mCherry in all reporter strains, we concluded that the *tfoY* transcript levels did not

398   significantly change in response to increased or decreased c-di-GMP levels (Fig. 4F). This

399   finding contradicted a recent study in *V. cholerae* that concluded that TfoY was induced at both

400   low and high intracellular concentrations of c-di-GMP and that this regulation occurred at the

401   translational and transcriptional levels, the latter due to activation by the c-di-GMP-dependent

402   transcription factor VpsR (Pursley *et al.*, 2018). We therefore constructed a similar dual

403   translational-transcriptional reporter strain in *V. cholerae* (Table S1) and tested this strain under

404   normal, increased, or decreased c-di-GMP levels. Using this approach, we were able to detect an

405   increase in the TfoY protein under low c-di-GMP levels compared to normal conditions, while

406   an increase in intracellular c-di-GMP did not result in higher TfoY protein levels (Fig. S2),

407   consistent with our previous findings (Metzger *et al.*, 2016). Importantly, we also did not observe

408   a change in the transcriptional reporter under high c-di-GMP conditions (Fig. S2), despite normal

409   VpsR function in the pandemic *V. cholerae* strain (A1552) used in this study (Yildiz *et al.*, 2001),

410   which confirmed the data presented above for *V. parahaemolyticus*. An explanation for this

411   discrepancy might be that we engineered the construct at the native locus of *tfoY* within the *V.*

412   *parahaemolyticus* or *V. cholerae* genomes, while Pursley *et al.* were unsuccessful in detecting

413   tagged TfoY variants using a similar approach. They therefore decided to construct a TfoY-GFP

- 17 -

414   translational-fusion-encoding reporter gene on a plasmid, which could change the expression

415   pattern compared to the gene's native locus. In addition, these authors performed their

416   experiments in a *Vibrio* polysaccharide mutant as parental strain (ΔvpsL), which is deficient in

417   biofilm formation (Pursley *et al.*, 2018), while our strain maintained a normal ability for this

418   process.

419   Collectively, we conclude that the lowered c-di-GMP levels observed in surface-grown *V.*

420   *parahaemolyticus* (Gode-Potratz *et al.*, 2011) might trigger TfoY induction, which, subsequently,

421   induces the T6SS1 and subsequently initiates motility, though the exact mechanism for the latter

422   remains to be discovered. The data provided here for *V. parahaemolyticus* therefore support our

423   previous suggestion that TfoY triggers a defensive escape reaction that might allow *Vibrio*

424   species to defend themselves against bacterial competitors and eukaryotic predators.

425   Interestingly, a recent study that profiled the gene expression of *V. parahaemolyticus* reported

426   increased *tfoY* expression (VP1028; falsely annotated as *tfoX* and therefore discussed as

427   competence regulator in this study) within infected infant rabbits when compared to *in vitro*

428   conditions (Livny *et al.*, 2014), suggesting that TfoY might also play a role *in vivo* and therefore

429   in pathogenesis.

430

431   **Conclusion**

432   In this study, we demonstrated that the link between the TfoX and TfoY regulatory proteins and

433   T6SSs is highly conserved among cholera and non-cholera *Vibrio* strains (Fig. 5), highlighting

434   the general importance of these regulators. Interestingly, TfoX induced at least one T6SS in

435   every tested species. Hence, we suggest that chitin-mediated TfoX production (Meibom *et al.*,

436   2005; Metzger and Blokesch, 2016) and the resulting coupling between T6SS-facilitated

437   neighbor killing and competence-mediated DNA uptake (Borgeaud *et al.*, 2015) might be an

438   ancient phenotype maintained in most *Vibrio* species. In contrast, the effect of TfoY on T6SS

- 18 -

439   turned out to be more versatile (Fig. 5). While the c-di-GMP-dependent inhibition of TfoY

440   translation seemed conserved, at least in the two tested *Vibrio* species (Fig. 4E and Fig. S2), the

441   regulons seems to have diverged in the different organisms. Indeed, while TfoY induces both

442   T6SSs in *V. alginolyticus* and one T6SS in *V. cholerae* and *V. parahaemolyticus* (out of two

443   T6SSs present in the latter bacterium), this regulator does not impact the activity of the T6SS of

444   *V. fischeri* under the tested conditions. Interestingly, several isolates of *V. fischeri,* other than the

445   squid isolate ES114*,* contain a secondary T6SS. This T6SS2 is located on a strain-specific

446   genomic island on the small chromosome and was recently shown to contribute to competitor

447   elimination within the host's light organ (Speare *et al.*, 2018). While we were unable to

448   genetically engineer the fish symbiont MJ11 strain for technical reasons, it is tempting to

449   speculate that this secondary T6SS2 of *V. fischeri* might be controlled by the TfoY protein.

450   Notably, Speare *et al*. proposed the possibility that this secondary T6SS2 was present in the

451   common ancestor of *V. fischeri* and related *Vibrio* species but was otherwise lost in niche-

452   adapted strains for which interbacterial killing is no longer advantageous. We extend this

453   hypothesis and speculate that niche-adapted vibrios might no longer require a TfoY-mediated

454   defensive response against either competing bacteria or grazing predators. Future studies are

455   required to address these interesting evolutionary hypotheses.

456

457   **Experimental procedures**

458   ***Bacterial strains, plasmids, and growth conditions***

459   The *V. cholerae, V. alginolyticus, V. parahaemolyticus,* and *V. fischeri* strains and plasmids used

460   in this study are listed in Table S1. *Escherichia coli* strains DH5α (Yanisch-Perron *et al.*, 1985),

461   TOP10 (Invitrogen), SM10λpir (Simon *et al.*, 1983), S17-1λpir (Simon *et al.*, 1983) and MFDpir

462   (Ferrieres *et al.*, 2010) were used for cloning purposes and/or served as donor in bacterial mating

463   experiments.

464   Unless otherwise stated, the *V. cholerae, V. alginolyticus, V. parahaemolyticus,* and *E. coli*

465   strains were grown aerobically in Lysogeny broth (LB; 10 g/L of tryptone, 5 g/L of yeast extract,

466   10 g/L of sodium chloride; Carl Roth) or on LB agar plates at 30°C or 37°C. *V. fischeri* strains

467   were cultured aerobically in LB salt (LBS) medium (10 g/L of tryptone, 5 g/L of yeast extract,

468   20 g/L of sodium chloride, 20 mM Tris-HCl [pH 7.5], 0.2% glycerol, adapted from (Dunlap,

469   1989; Graf *et al.*, 1994)) or LBS agar plates at 28°C. LB/LBS motility plates had less agar (0.3%)

470   than standard LB/LBS agar plates (1.5%). The following were added if required at the given

471   concentrations: arabinose (0.02% or 0.2%), diaminopimelic acid (DAP; 0.3 mM), or the

472   antibiotics kanamycin (75 µg/ml), gentamicin (50 µg/ml), and chloramphenicol (2.5 µg/ml or 5

473   µg/ml). L-arabinose-supplemented medium was used for the expression of *tfoX* or *tfoY* under the

474   control of the P$_{BAD}$ promoter. DAP was added as an essential growth supplement for *E. coli*

475   strain MFDpir (Ferrieres *et al.*, 2010). Medium without DAP was used to counter-select MFDpir

476   strains after tri-parental mating with *V. fischeri*. For the *E. coli* counter-selection after tri-parental

477   mating with *V. cholerae, V. alginolyticus,* and *V. parahaemolyticus,* Thiosulfate Citrate Bile

478   Salts Sucrose (TCBS) agar plates were used and prepared following the manufacturer's

479   instructions (Sigma-Aldrich/Fluka, Buchs, Switzerland). Marine broth 2216 (BD Difco[TM] 2216)

480   was used to isolate single colonies of *V. alginolyticus* and *V. parahaemolyticus* after *E. coli*

481   counter-selection.

482

483   ***Genetic engineering of strains and plasmids***

484   DNA manipulations were performed according to standard molecular biology-based protocols

485   (Sambrook *et al.*, 1982). Enzymes were purchased from the listed companies and were used as

486   recommended by the manufacturer: Pwo polymerase (Roche), Taq polymerase (Promega),

487   restriction enzymes (New England Biolabs). Following initial screening by PCR (using bacterial

488   cells as templates), genetically engineered strains and plasmids were verified by Sanger

489   sequencing (Microsynth, Switzerland).

490   *V. cholerae* strains were genetically modified using both a gene-disruption method based on

491   the counter-selectable plasmid pGP704-Sac28 (Meibom *et al.*, 2004) or the TransFLP gene

492   disruption method previously described by our group (De Souza Silva and Blokesch, 2010;

493   Blokesch, 2012b; Borgeaud and Blokesch, 2013). This transformation-based genetic engineering

494   technique was used to replace the *tfoY* gene by a translational and transcriptional *tfoY-*

495   *mCherry::gfp* fusion at the natural locus on the *V. cholerae* A1552 chromosome. Plasmid

496   pGP704-Sac-Kan (see below), derived from pGP704-Sac28, was used for genetic modifications

497   of *V. parahaemolyticus*.

498   To construct the counter-selectable plasmid pGP704-Sac-Kan, the *aph* gene was amplified

499   from a kanamycin-resistance gene (*aph*)-carrying transposon using primers with overhanging

500   *Ssp*I and *Bsa*I restriction sites. A restriction digestion was performed on both the PCR product

501   and the vector pGP704-Sac28, which were subsequently ligated, to replace the *bla* gene of

502   pGP704-Sac28 with the *aph* gene, resulting in plasmid pGP704-Sac-Kan (Table S1).

503   All pBAD-derived plasmids harboring *tfoX-strep* or *tfoY-strep* genes from different *Vibrio*

504   species were constructed in the following way: The genes were amplified with *StreptagII-*

505   encoding primers using gDNA of the respective parental *Vibrio* strain as a template. The

506   restriction-enzyme-digested PCR product was subsequently cloned into plasmid

507   pBAD/MycHisA (Table S1). The resulting plasmids served as templates for fragments

508   containing *araC*, the arabinose-inducible promoter P_{BAD}, and the given *tfoX*, *tfoY* gene, which

509   were subcloned into the mini-Tn7-containing delivery plasmids (Table S1). The plasmid

510   pGP704-Tn-Cm^R is a derivative of pGP704-mTn7-minus-SacI (Nielsen *et al.*, 2006), where the

511   *aacC1* gene within the mini-Tn*7* was replaced by the *cat* gene. To this end, the PCR-amplified

512   *cat* gene (including its promoter) of pBR-FRT-Cat-FRT2 (Metzger *et al.*, 2016) was cloned into

513   pGP704-mTn*7*-minus-SacI, which was digested using restriction enzymes *Sbf*I and *EcoR*V. For

514   the insertion of this mini-Tn*7* transposon into the *Vibrio spp.* chromosomes, a tri-parental mating

515   strategy was employed (Bao *et al.*, 1991). The donor plasmids are indicated in Table S1.

516

517   ***Natural transformation assay***

518   Natural transformation assays in liquid were performed with minor modifications to the previous

519   protocol (Lo Scrudato and Blokesch, 2012). This assay is chitin-independent and uses strains

520   carrying an arabinose-inducible copy of *tfoX-strep* or *tfoY-strep* (both from *V. cholerae, V.*

521   *alginolyticus, V. parahaemolyticus*, or *V. fischeri*) on the chromosome. *V. cholerae* strains were

522   pre-grown overnight in LB medium and further grown in the presence of the arabinose inducer

523   up to an optical density at 600 nm (OD_{600}) of 1.0. At this point, 0.5 ml of the cultures were

524   supplemented with 1 µg of genomic DNA. For all natural transformation assays, the genomic

525   DNA of A1552-lacZ-Kan (Marvig and Blokesch, 2010) served as transforming material. Cells

526   were further incubated under shaking conditions at 30°C for 4 hours. Serial dilutions were

527   spotted on LB to count the total number of cells and on kanamycin-containing LB agar plates to

528   select the transformants. Transformation frequencies were calculated as the number of colony-

529   forming units (CFUs) of the transformants divided by the total number of CFUs. Averages of at

530   least three biologically independent experiments are provided. For statistical analyses, the data

531   were log-transformed and significant differences were determined by the two-tailed Student's t-

532   test. When no transformants were recovered, the value was set to the detection limit to allow for

533   statistical analysis.

534

535

536   *Gene expression analysis by quantitative reverse transcription PCR (qRT-PCR)*

537   Quantitative reverse transcription PCR (qRT-PCR)-based transcript scoring in *V. cholerae, V.*

538   *alginolyticus, V. parahaemolyticus*, and *V. fischeri* was performed following a previously

539   established protocol (Lo Scrudato and Blokesch, 2012). Strains with and without an arabinose-

540   inducible copy of specified genes (*tfoX-strep* or *tfoY-strep*) were grown for 6 hours in 2.5 ml LB

541   or LBS supplemented with 0.2% arabinose. Cultures (2 ml) were processed for RNA isolation

542   and subsequent cDNA synthesis. Relative gene expression values were normalized against the

543   *gyrA* transcript levels. Fold changes were determined using the relative expression values of the

544   induced strains divided by the values of the parental WT strain (as specified for each inducible

545   construct in the figures). Averages of at least three biologically independent experiments (±

546   standard deviation) are provided.

547

548   *Interbacterial killing assay using E. coli as prey*

549   The *E. coli* killing assay was performed following a previously established protocol with minor

550   adaptions (Borgeaud *et al.*, 2015). The *E. coli* prey cells and the given predator cells were mixed

551   at a ratio of 10:1 and spotted onto membrane filters on pre-warmed LB and/or LBS agar plates (±

552   0.2% ara). After 4 h of incubation at 28°C (predator: *V. fischeri*) or 30°C and 37°C (as indicated

553   for the predators *V. cholerae, V. alginolyticus*, *and V. parahaemolyticus*), bacteria were

554   resuspended and serial dilutions were spotted onto antibiotic-containing LB agar plates to

555   enumerate the CFUs (shown as CFU/ml). Arabinose-uptake-deficient *E. coli* TOP10 and its

556   derivative TOP10-TnKan served as prey. Significant differences were determined by a two-tailed

557   Student's t-test on log-transformed data of at least three biological replicates. If no prey cells

558   were recovered, the value was set to the detection limit to allow statistical analysis.

559

560   *Motility assay*

561   The motility of the *Vibrio* species was assessed by spotting 2 µl of the respective overnight

562   culture onto freshly prepared LB and/or LBS motility agar plates (containing 0.3% agar) with or

563   without 0.2% arabinose. Following the incubation at 30°C for 6h for *V. cholerae, V.*

564   *parahaemolyticus*, and *V. alginolyticus*, or at room temperature for 7h for *V. fischeri*, the

565   diameters of the bacterial swarming were determined. The motility induction was calculated by

566   dividing the swarming diameter of induced versus uninduced strains. The averages of at least

567   three independent experiments (± standard deviation) are provided. For statistical analyses, a

568   two-tailed Student's t-test was performed.

569

570   *SDS-PAGE and western blotting*

571   Cell lysates were prepared as described previously (Metzger *et al.*, 2016). In brief, after

572   cultivation with or without arabinose for 3 or 6 hours, bacterial cell pellets were resuspended in

573   Laemmli buffer, adjusting for the total number of bacteria according to the OD_{600} values.

574   Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

575   and western blotted as described (Lo Scrudato and Blokesch, 2012). Primary antibodies against

576   Hcp (Eurogentec; (Metzger *et al.*, 2016)), GFP (Roche, Switzerland), and mCherry (BioVision,

577   USA distributed via LubioScience, Switzerland) were used at 1:5,000 dilutions, and *E. coli*

578   Sigma70 (BioLegend, USA distributed via Brunschwig, Switzerland) was used at a 1:10,000

579   dilution. Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP (both diluted

580   1:20,000; Sigma-Aldrich, Switzerland) served as secondary antibodies. Lumi-Light[PLUS] western

581   blotting substrate (Roche, Switzerland) was used as an HRP substrate and the signals were

582   detected using a ChemiDoc XRS+ station (BioRad).

583

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593

Author contributions

595   Conception, design and analysis: L.C.M, N.M., and M.B.; performed research: L.C.M, N.M.,

596   C.S., E.J.C., and M.B; wrote the manuscript: L.C.M. and M.B. with input from N.M..

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795   1673.

796   **Figure legends**

797   **Figure 1: TfoX and TfoY proteins from diverse *Vibrio* species are functional in *V. cholerae.***

798   (A) Phylogenetic tree and protein sequence conservation of TfoX and TfoY across *Vibrio* species.

799   The phylogenetic tree was generated on phlyot.biobyte.de, a phylogenetic tree generator, based

800   on NCBI taxonomy. The protein sequences of TfoX and TfoY were aligned using ClustalOmega,

801   and subsequently modified with Jalview. Identical residues are highlighted in shades of blue

802   (above threshold of 65%). (B-J) Chromosomally-located *tfoX* and *tfoY* from cholera (*V. cholerae*)

803   and non-cholera *Vibrio* species (*V. parahaemolyticus*, *V. alginolyticus,* and *V. fischeri*) were

804   expressed under the control of the arabinose-inducible P_{BAD} promoter in *V. cholerae*. The color

805   code (TfoX-induced, green; TfoY-induced, blue) is used throughout the graphs. The WT strain

806   lacking an inducible copy of *tfoX* or *tfoY* served as the negative control. The fold change (*tfoX*-

807   or *tfoY*-induced over parental WT strain) of relative gene expression of (B, G) T6SS genes or (E)

808   representative competence genes. (C, H) Detection of Hcp protein produced by TfoX- and TfoY-

809   expressing cells. Cells were grown in the absence or the presence of the inducer (arabinose) as

810   indicated below the image. Detection of σ70 served as a loading control. (D, I) Interspecies

811   killing assay with *E. coli* as prey. *V. cholerae* harboring different inducible versions of (D) *tfoX*

812   or (I) *tfoY* were co-cultured with the prey on LB agar plates supplemented with arabinose. The

813   survival of the prey was determined on selective LB agar plates and is depicted as CFU per ml.

814   (F) Natural transformation using genomic DNA is maintained in a *V. cholerae* strain expressing

815   *tfoX* from non-cholera vibrios but that is non-functional upon *tfoY* expression. The indicated

816   strains were grown under inducible conditions, and the genomic DNA of A1552-lacZ-Kan

817   served as the transforming material. Transformation frequencies reflect the number of

818   transformants divided by the total number of CFUs. < dl, below detection limit. (J) Motility was

819   scored on soft agar with and without arabinose as an inducer. The motility phenotype was

820   quantified as the ratio between the induced and uninduced conditions, as shown on the Y-axis.

821   Abbreviations: V.c., *Vibrio cholerae* A1552, V.p., *Vibrio parahaemolyticus* RIMD2210633; V.a.,

822   *Vibrio alginolyticus* 12G01; V.f., *Vibrio fischeri* ES114. Bar plots represent the average of at

823   least three independent biological replicates (± SD). Statistical significance is indicated (*$p <$

824   0.05; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

825

826   **Figure 2: TfoX but not TfoY induce *V. fischeri*'s T6SS.** The effect of TfoX (green) and TfoY

827   (blue) production in *V. fischeri* ES114 was scored by qRT-PCR, and the relative expression

828   values comparing uninduced versus induced conditions are indicated for representative (A) T6SS

829   or (C) competence genes. (B) TfoX induction in *V. fischeri* leads to interbacterial killing of *E.*

830   *coli* prey cells. *V. fischeri* strains were co-cultured with *E. coli* for 4 hours at 28°C on plain LBS

831   agar plates supplemented with arabinose to induce the arabinose-inducible copy of *tfoX* or *tfoY*

832   (as indicated below the graph). The values for the recovered prey are indicated on the Y-axis. (D)

833   TfoY fosters motility in *V. fischeri.* Quantification of the motility phenotype of TfoX- or TfoY-

834   induced bacteria normalized by the uninduced conditions. The WT strain without any inducible

835   gene served as a control. (A-D) Bar plots represent the average of at least three independent

836   biological replicates (± SD). Statistical significance is indicated (*$p < 0.05$; **p < 0.01).

837

838   **Figure 3: TfoY is a universal T6SS inducer in *V. alginolyticus*, while TfoX is specific for**

839   **T6SS2.** (A, B) Interspecies killing assay of *V. alginolyticus* with *E. coli* as prey. *V. alginolyticus*

840   strains were co-cultured with *E. coli* prey for 4 hours at (A) 37°C (B) or 30°C on plain LB agar

841   supplemented with arabinose to induce *tfoX* or *tfoY* (as indicated below the graph). The recovery

842   of the prey is indicated as CFU/ml on the Y-axis. (C) TfoX-induces competence genes in *V.*

843   *alginolyticus.* The relative expression of representative competence genes was scored in *tfoX*-

844   induced and *tfoX*-uninduced strains, and the fold change between the values is indicated on the

845   Y-axis. (D) Motility ratio between uninduced and induced *V. alginolyticus* strains is indicated.

846   Details as in Fig. 2D. Abbreviations: WT, *V. alginolyticus* strain 12G01; ΔT6SS1, 12G01Δhcp1;

847   ΔT6SS2, 12G01Δhcp2; ΔT6SS1ΔT6SS2, 12G01Δhcp1Δhcp2. Bar plots represent the average of

848   at least three independent biological replicates (± SD). Statistical significance is indicated (*$p <$

849   0.05; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

850

851   **Figure 4: TfoX and TfoY each induce a dedicated T6SS in *V. parahaemolyticus*.** (A, E)

852   Interspecies killing of *E. coli* by *V. parahaemolyticus*. *V. parahaemolyticus* strains (WT, T6SS1-,

853   T6SS2-, T6SS1/T6SS2-minus or *opaR*-minus) were co-cultured with *E. coli* prey for 4 hours at

854   30°C on LB agar plates supplemented with arabinose to induce *tfoX* or *tfoY* (as indicated below

855   the graph). The recovery of the prey is indicated on the Y-axis. (B) Basal T6SS activity is

856   independent of TfoX and TfoY. WT *V. parahaemolyticus* or its *tfoX*-, *tfoY*-, or *tfoX/tfoY*-minus

857   variants were tested for their ability to reduce the number of *E. coli* prey cells. The T6SS double

858   mutant (ΔT6SS1ΔT6SS2) served as a control. (C) Fold change (*tfoX*-induced over the WT

859   parental strain) of relative gene expression of representative competence genes. (D)

860   Quantification of TfoX- or TfoY-induced motility phenotypes. Details as in Fig. 2D. (F) TfoY is

861   produced under low c-di-GMP conditions in *V. parahaemolyticus*. Detection of TfoY-sfGFP and

862   mCherry by western blotting in *cdpA*- or *vdcA*-inducible reporter strains that contain increased or

863   decreased intracellular c-di-GMP levels, as indicated by the arrows. The transposon-less or

864   empty transposon-carrying reporter strain as well as the parental WT strain served as controls.

865   Detection of σ70 served as a loading control. RBS, ribosome-binding site. Abbreviations: WT, *V.*

866   *parahaemolyticus* POR1 (RIMD2210633 derivative); ΔT6SS1, POR1Δhcp1; ΔT6SS2,

867   POR1Δhcp2; ΔT6SS1ΔT6SS2, POR1Δhcp1Δhcp2. Bar plots represent the average of at least

868   three independent biological replicates (± SD). Statistical significance is indicated (*$p < 0.05$;
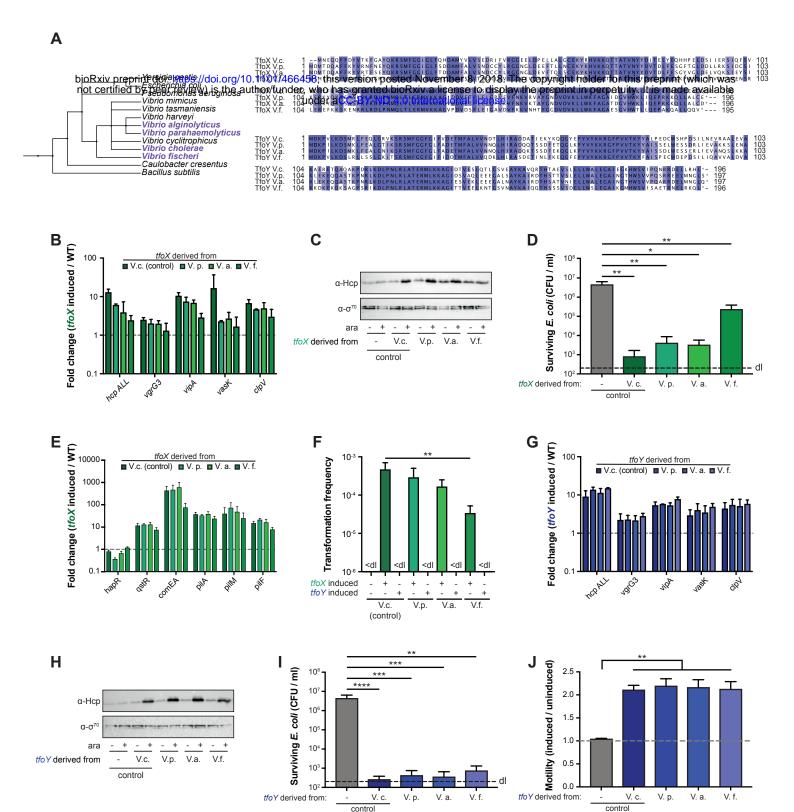
869   **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

870

871

Figure 5: Summary scheme of TfoX- and TfoY-mediated T6SS induction in cholera and non-cholera *Vibrio* species. The T6SS clusters of *V. cholerae, V. fischeri, V. alginolyticus,* and *V. parahaemolyticus* are indicated (scale corresponds to 5 kbp as indicated). Arrows indicate positive regulation by the respective regulator TfoX or TfoY.

876

Supporting Information

Additional Supporting Information can be found in the online version of this article at the publisher's webpage: XXX

880

Table S1: Bacterial strains and plasmids used in this study.

882

883

Supporting figure legends

Figure S1: TfoX- and TfoY-induced T6SS production is conserved in pandemic *V. cholerae* strains. Interspecies killing assay between diverse *V. cholerae* strains and *E. coli* as prey. The *V. cholerae* O1 El Tor strains tested are as follows: A1552, N16961rep (with repaired frameshift mutation in *hapR*), C6709, E7946, and P27459 as indicated below the graph. Co-culturing with *E. coli* occurred on LB agar plates supplemented with arabinose to induce *tfoX* or *tfoY* where indicated. The parental strains without inducible copies of the regulatory genes served as a control. Prey recovery is indicated as CFU/ml on the Y-axis. Bar plots represent the average of three independent biological replicates (± SD). Statistical significance is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

894

895 **Figure S2: TfoY production is translationally but not transcriptionally controlled by c-di-**

896 **GMP in *V. cholerae*.** *V. cholerae* reporter strains carrying a gene encoding for a translational

897 fusion between TfoY and mCherry (*tfoY-mCherry*) with or without a downstream transcriptional

898 reporter gene (*gfp*) at the gene's native chromosomal locus were genetically manipulated to

899 insert inducible copies *vdcA* or *cdpA* into their genome (inside a mini-Tn7 transposon). Cells

900 were then grown under inducible conditions to increase or decrease intracellular c-di-GMP

901 concentrations, as shown by the arrows above the images. Detection of TfoY-mCherry and GFP

902 occurred through western blotting. The reporter strain lacking the transposon as well as the

903 parental WT served as controls. Detection of σ70 served as a loading control.

Fig. 1

A
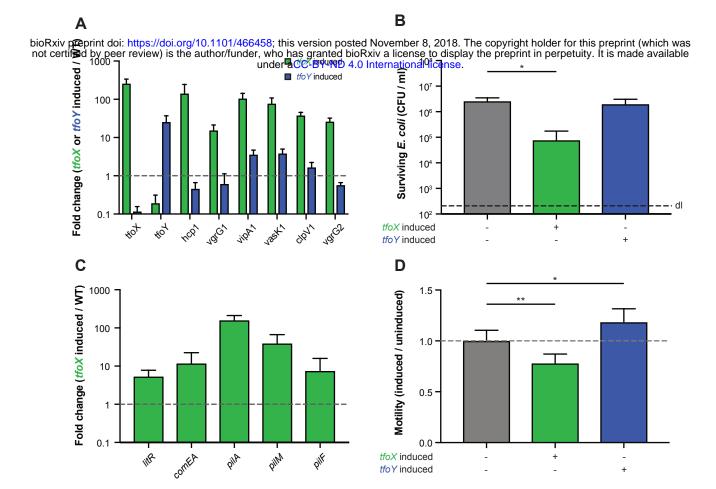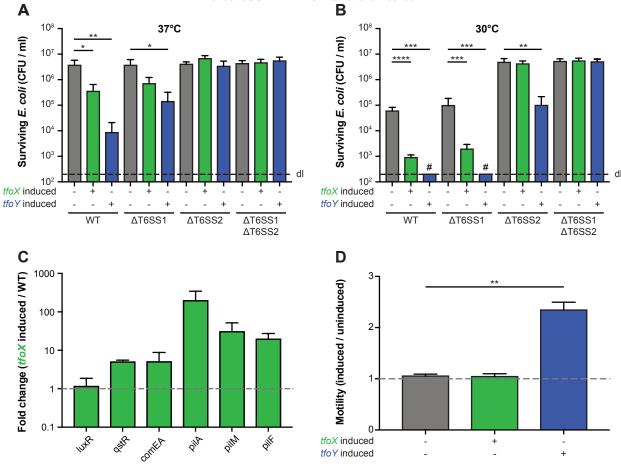
B

C

D

Fig. 2

Fig. 3

Fig. 4

Fig. 5